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Research Article

Up-regulated ferritin in periodontitis promotes inflammatory cytokine expression in human periodontal ligament cells through transferrin receptor via ERK/P38 MAPK pathways

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Objective: Ferritin, an iron-binding protein, is ubiquitous and highly conserved; it plays a crucial role in inflammation, which is the main symptom of periodontitis. Full-length cDNA library analyses have demonstrated abundant expression of ferritin in human periodontal ligament. The aims of the present study were to explore how ferritin is regulated by local inflammation, and to investigate its functions and mechanisms of action in the process of periodontitis. Methods: Human gingival tissues were collected from periodontitis patients and healthy individuals. Experimental periodontitis was induced by ligature of second molars in mice. The expression of ferritin light polypeptide (FTL) and ferritin heavy polypeptide (FTH) were assessed by immunohistochemistry. Meanwhile, after stimulating human periodontal ligament cells (HPDLCs) with *P. gingivalis*-lipopolysaccharide (LPS), interleukin (IL)-6, and tumor necrosis factor-α (TNF-α), the expression of FTH and FTL were measured. Then, IL-6 and IL-8 were measured after incubation with different concentrations of apoferritin (iron-free ferritin) and several intracellular signaling pathway inhibitors, or after knockdown of the transferrin receptor. Results: Both FTH and FTL were substantially higher in inflamed periodontal tissues than in healthy tissues. The location of the elevated expression correlated well with the extent of inflammatory infiltration. Moreover, expression of FTH and FTL were enhanced after stimulation with *P. gingivalis*-LPS, IL-6, TNF-α. Apoferritin induced the production of IL-6 and IL-8 in a dose-dependent manner partly through binding to the transferrin receptor and activating ERK/P38 signaling pathways in HPDLCs. Conclusions: Ferritin is up-regulated by inflammation and exhibits cytokine-like activity in HPDLCs inducing a signaling cascade that promotes expression of pro-inflammatory cytokines associated with periodontitis.

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Introduction

Periodontitis is a chronic inflammatory disease characterized by the destruction of periodontal supporting tissues [1,2]. Periodontal ligament is a specialized connective tissue situated between the roots of teeth and inner wall of alveolar bone, and has a role of maintaining and supporting the teeth [3]. Periodontal

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ligament cells (PDLCs) are the main type of cells in periodontal ligament (PDL) and have a few characteristics of immune cells [4].

The pathogenic molecular mechanisms of periodontitis remain largely unknown. The presence of periodontopathic bacteria is required but not sufficient for initiation of periodontitis [5]. The host immune-inflammatory responses also play an important role in the initiation and progression of periodontitis [6]. PDLCs secrete pro-inflammatory cytokines that sustain local inflammation and cause tissue breakdown [7]. IL-6 is a pro-inflammatory cytokine coming from host immune system and promotes alveolar bone loss [8,9]. IL-8 is a chemoattractant and can activate neutrophils, lymphocytes, and macrophages excitation [10,11]. Previous studies showed that ERK and P38 MAPK pathways were the common pathways involved in IL-6 and IL-8 production in HPDLCs [12–14]. However, factors that affect the reaction of PDLCs are still unclear.

Ferritin, an iron-binding protein, is ubiquitous, highly conserved, and composed of two subunits, the ferritin light chain (FTL, 19 kDa) and the ferritin heavy chain (FTH, 21 kDa) [15–19]. Ferritin stores iron and prevents infective agents from binding iron. Within cells, ferritin has anti-inflammatory properties [20,21] and its expression is regulated by pro-inflammatory cytokines [20,22]. Ferritin is an acute-phase reactant, and it is elevated in chronic infection and inflammation [23,24]. However, the precise mechanisms of ferritin release and its actions in the serum and tissues are unclear [13].

In a previous study, we found that ferritin is predominantly expressed in periodontal ligament tissues and regulates the mineralization of PDLCs [25,26]. Serum, salivary, and GCF ferritin are elevated in patients with chronic periodontitis [27–29]. However, the specific correlation between ferritin and periodontitis has few reports. Therefore, the aims of the present study are to explore the influence of periodontitis on ferritin localization and expression, and to investigate the effects of ferritin on the behavior of PDLCs.

Materials and methods

Ethics statement

The present study was approved by the Review Board and the Ethics Committee of Peking University Health Science Center (IRB00001052-08010). All clinical specimens were obtained from patients, who provided written informed consent to use their tissues for research purposes.

Study population

A total of 20 participants were recruited from the Department of Periodontology, Peking University Hospital of Stomatology, China. They included 10 healthy individuals and 10 patients diagnosed with periodontitis. All individuals were examined to determine their clinical periodontal status. Periodontal examination included periodontal probing depth (PD) and bleeding index (BI) using a Williams periodontal probe at six sites on each tooth. The tissue specimens were taken from healthy individuals who received crown-lengthening surgery and from patients with periodontitis who received tooth-extraction and site preservation. The harvested gingival tissues were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (H&E).

Experimental animals and tissue samples

The study protocol was approved by the Experimental Animal Welfare Ethics Branch of Peking University Biomedical Ethics Committee (Protocol LA2013-32). Male C57BL/6 mice (8–10 weeks old) were randomly divided into groups. In the periodontitis groups (n=10), a 5/0 cotton ligature was placed around the gingival margin of the second molars for 1, 3, or 7 days. And the mice in the control group were unligated. Mice were killed 1, 3, or 7 days later after ligating. Upper jaw samples were excised and fixed in 4% paraformaldehyde (pH 7.4) overnight at room temperature. Then, the samples were demineralized in buffered 20% EDTA at 37°C for 14 days, then embedded and cut on a microtome set at 5 μm. Some sections from each sample were stained with H&E.

Immunohistochemical staining of human gingival samples and mouse periodontal samples

After deparaffinization of samples with xylene and rehydration with ethanol, endogenous peroxidase was inactivated by incubation with 3% H_2O_2 at room temperature for 20 min. Antigen retrieval was achieved by trypsin digestion at 37°C for 20 min. Then, the specimens were blocked with 10% normal goat serum at room temperature for 30 min and incubated with primary antibodies against FTH, FTL (Santa Cruz, CA, U.S.A.), and COX2 (Cell Signaling Technology, MA, U.S.A.) at 4°C overnight. After washing three times with phosphate-buffered saline, the locations of FTH, FTL, and COX2 were visualized using an immunohistochemistry kit and a DAB detection kit (Zhongshan

Table 1 Primers used for qRT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAAT
FTH	CAGGTGCGCCAGAACTACCA	CCACATCATCGCGGTCAAAG
FTL	ACCATGAGCTCCCAGATTCGTC	CACATCATCGCGGTCGAAATAG
IL-6	GTGAGGAACAAGCCAGAC	TACATTTGCCGAAGAGCC
IL-8	CCTCTGCGGGACTCAACAAC	TAAAGGGGGCTGGATAAGCAT

Golden Bridge Biotechnology, Beijing, China). Sections were finally mounted and images were captured on a light microscope (BX51/DP72, Olympus, Tokyo, Japan).

Cell culture and stimulation

Primary normal HPDLCs were obtained from explant cultures. Periodontal ligament explants were harvested from the middle third of extracted wisdom teeth or premolar roots extracted for orthodontic treatment. HPDLCs from 7 donors who had no periodontal or tooth diseases were cultured as previously described [30]. They were used for experiments in passages 4–8. We cultured HPDLCs in six-well plates for mRNA examination and protein detection.

To explore the effects of various factors on ferritin production, HPDLCs were treated with lipopolysaccharide (LPS) derived from *Porphyromonas gingivalis* (*P. gingivalis*) (InvivoGen, San Diego, CA, U.S.A.), interleukin (IL)-6, and tumor necrosis factor-α (TNF-α) (Peprotech, Rocky Hill, NJ, U.S.A.) for mRNA and protein detection. Meanwhile, to determine the effects of ferritin on IL-6 and IL-8 production, HPDLCs were treated with 0, 1, 10, 100, 500, or 1000 ng/ml apoferritin (Sigma–Aldrich, St. Louis, MO) for 6 h for mRNA and protein assessment.

Inhibitors of ERK (U260), P38 MAPK (SB203580), c-Jun N-terminal kinase (JNK) (SP600125) were used to explore the signaling pathways involved in the effect of ferritin on IL-6 and IL-8 production in HPDLCs. After pre-treatment with an inhibitor for 1 h each, HPDLCs were stimulated with 100 ng/ml apoferritin for 6 h before mRNA and protein assessment.

RNA extraction and real-time PCR analysis

Total RNA was extracted from treated HPDLCs using TRIzol (Invitrogen, Carlsbad, CA, U.S.A.). Then, RNA was used for cDNA synthesis with a Reverse Transcription System (Toyobo, Osaka, Japan). The synthetic cDNA was mixed with Power SYBR Green PCR Master Mix (Roche, Indianapolis, IN, U.S.A.) and gene-specific primers. The nucleotide sequences of the primers are listed in Table 1. Real-time PCR was performed using a 7500 Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, U.S.A.). The amplification conditions consisted of an initial 10 min denaturation step at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and elongation at 72°C for 30 s. The relative expression levels of targets were assessed using the comparative $2^{\Delta\Delta C}{}_{t}$ method.

Enzyme-linked immunosorbent assay (ELISA)

HPDLCs were treated with 0, 1, 10, 100, 500, 1000 ng/ml apoferritin for 6 h and 0, 100, 250, 500, 1000, 2000 ng/ml *P. gingivalis*-LPS for 6 h. The culture supernatant was collected, centrifuged, and stored at −80°C. The concentrations of IL-6, IL-8, or ferritin secreted by HPDLCs were measured using ELISA kits (Beijing Qisong Biotech Company, Beijing, China).

Western blots

Cells were harvested and lysed with RIPA buffer containing 1% PMSF (Sigma–Aldrich, St. Louis, MO). After centrifugation at 12000 ***g*** for 0.5 h, the supernatant was collected. Pierce BCA Protein Assay kits (Thermo Scientific, Rockford, U.S.A.) were used to determined protein concentrations. Thirty micrograms of protein from each sample was separated on 12% SDS–PAGE and then transferred to polyvinylidene difluoride membranes (Merck Millipore, U.S.A.). The membranes were blocked for 1 h in TBST containing 5% non-fat dried milk. After washing, the membranes were incubated in the primary antibodies at 4°C overnight. Primary antibodies against FTH (Abcam, Cambridge, MA, U.S.A.), FTL (Santa Cruz, CA, U.S.A.), P38, p-P38, ERK, p-ERK, JNK, p-JNK and GAPDH (Cell Signaling Technology, MA, U.S.A.) were diluted 1:1000. After washing three times with TBST for 15 min each, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies

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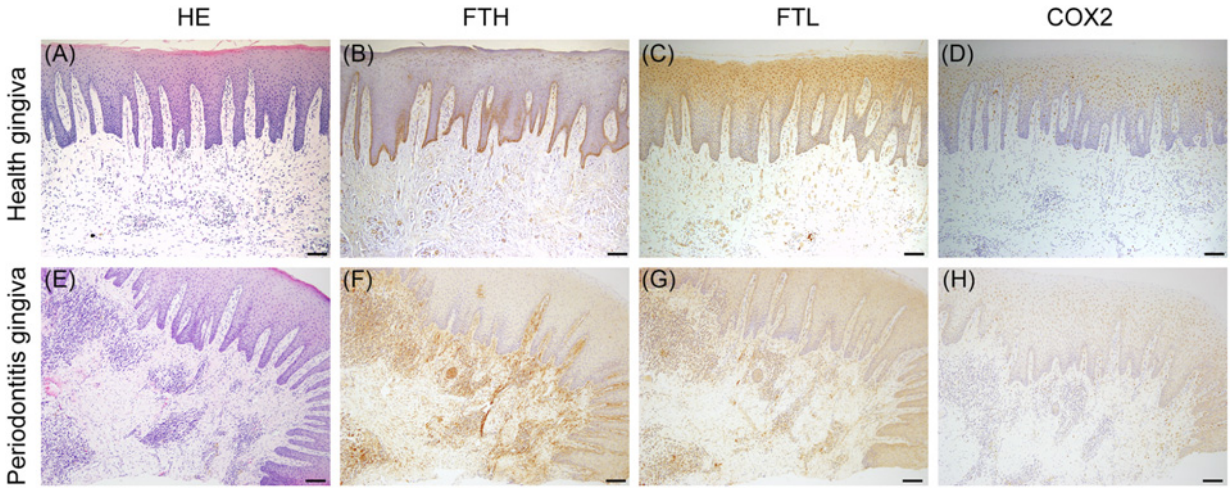

Figure 1. Immunohistochemical localization and expression of FTH and FTL in human gingiva

The staining for FTH (**B**), FTL (**C**), and COX2 (**D**) in healthy gingival tissue is positive. Note that FTH was evident in the basal layer, while FTL was in the spinous layer (magnification, 10×). H&E staining showed that gingival tissue affected by periodontitis (**E**) had more infiltration than healthy tissue (**A**). The expression of FTH (**F**), FTL (**G**), and COX2 (**H**) were stronger in the inflamed tissue. FTH disappeared from basal layer (magnification, 10×). Scale bars: 100 μm.

(Zhongshan Golden Bridge Biotechnology, Beijing, China) at room temperature for 60 min and then assessed using an ECL kit (CWBIO, Beijing, China). ImageJ software (https://imagej.nih.gov/ij/) was used to quantify band intensity.

RNA Interference of TfR-1

After HPDLCs had grown to 60–80% confluence in six-well plates, they were transfected with transferrin receptor (TfR-1) small-interfering RNA or a non-silencing control siRNA (10 nM, GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) according to the instructions of the manufacturer. Twenty-four hours after transfection, cells were treated with 100 ng/ml apoferritin for 6 h. The levels of IL-6 and IL-8 were determined by ELISA and qPCR.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 6 software. Results are presented as mean ± SEM. Statistical analyses were carried out using Student's *t*-test for testing two groups and one-way ANOVA for testing multiple groups. A value of $P<0.05$ was considered statistically significant. Results were acquired at least in triplicate in three separate experiments.

Results

Expression of ferritin (FTH and FTL) are increased in inflammatory tissues of periodontitis

Immunohistochemical staining of healthy gingival samples showed that FTL was distributed in the stratum spinosum and vessels (Figure 1C), and FTH was distributed in the basal cell layer and vessels (Figure 1B). Immunohistochemical staining of inflammatory gingival samples showed that FTL and FTH were up-regulated in connective tissue in areas of inflammatory cell infiltration (Figure 1F,G) and FTH disappeared from the basal cell layer (Figure 1F).

To confirm the result that ferritin is up-regulated in inflamed tissues, we generated an animal model of experiment periodontitis. Micro-computed tomography (CT) and H&E examination confirmed the establishment of periodontitis in C57BL/6 mice. Micro CT analysis showed absorption of alveolar bone and H&E staining showed massive inflammatory cell infiltration of tooth-supporting tissues. Furthermore, immunohistochemical staining showed that FTH and FTL were expressed widely in the unligated periodontal tissues, in agreement with the results of previous study [10]. Gingival epithelium, periodontal ligament tissue, and gingival connective tissue are all positively stained (Figure 2A,E). Meanwhile, the locations of positive FTH and FTL staining in the ligated tissues were exactly the same

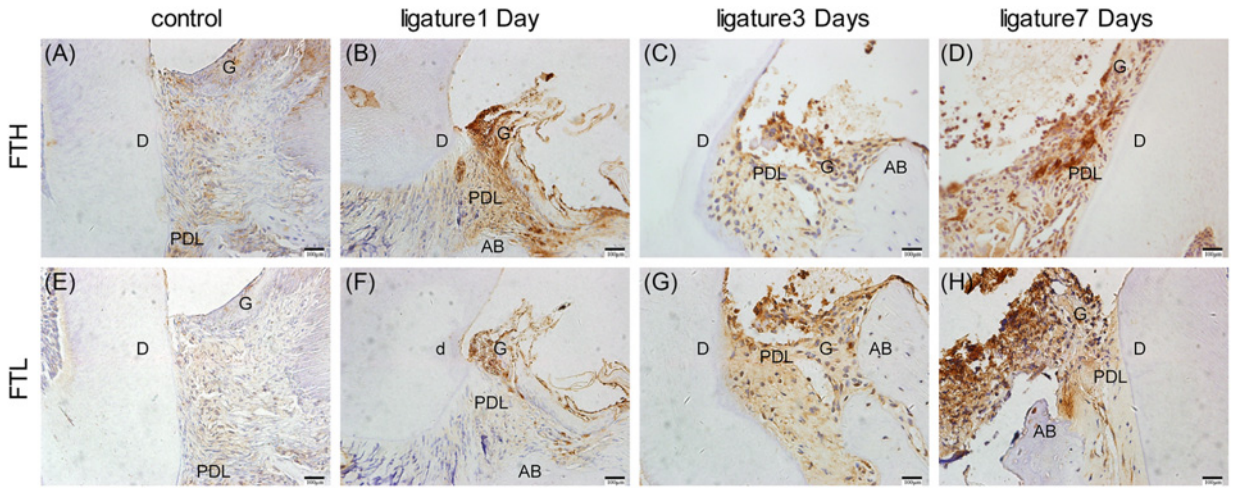

Figure 2. Immunohistochemical localization and expression of FTH and FTL in mice's periodontal tissues of periodontitis

FTH and FTL staining increased gradually over time in periodontal tissue of mice with experimental periodontitis. Gingival epithelial cells were destroyed, and infiltration was more evident at later times. The expression of FTH (**A**, **B**, **C**, and **D**) and FTL (**E**, **F**, **G**, and **H**) also increased gradually. G: gingiva, D: dentin, PDL: periodontal ligament, AB: alveolar bone. Magnification, 10×. Scale bars: 100 μm.

as in the unligated tissues. However, the intensity of positive staining became significantly stronger along with the extent of inflammatory infiltration (Figure 2).

P. gingivalis-LPS promotes ferritin expression and secretion in HPDLCs

Porphyromonas gingivalis is the main periodontal pathogen in periodontitis patients [14]. After stimulation with *P. gingivalis*-LPS, the levels of ferritin mRNA and protein rose to a peak at 24 h with 100 ng/ml (Figure 3A–D), while they peaked at 500 ng/ml for 3 h (Figure 3E–H). The secretion of ferritin also was promoted by *P. gingivalis*-LPS at the level of 2000 ng/ml for 6h (Figure 3 I).

IL-6 and TNF-α promote ferritin (FTH and FTL) expression in HPDLCs

IL-6 and TNF-α, the most important pro-inflammatory host factors, are clearly elevated in the gingival crevicular fluid (GCF) of periodontitis [15]. Our results showed that IL-6 at 10 ng/ml for 6 h increased the expression of FTH (Figure 4A,B) and FTL (Figure 4C,D). At the same time, TNF-α at 10 ng/ml for 30 min promoted FTH (Figure 4E,F) and FTL (Figure 4G,H) expression. These data demonstrate that the exogenous factor, *P. gingivalis*-LPS, is not the only factor in promoting ferritin expression; endogenous factors, such as IL-6 and TNF-α, also aggravate ferritin expression.

Ferritin induces IL-6 and IL-8 production in HPDLCs

HPDLCs were treated with apoferritin (iron-free ferritin) after serum-starvation in DMEM without FBS for 12 h. After apoferritin stimulation, the expression of IL-6 and IL-8 mRNA and protein levels were dose-dependently up-regulated at 6 h compared with the control (Figure 5). Extracellular ferritin induces IL-6 and IL-8 production in HPDLCs via an iron-independent pathway.

Ferritin activates intracellular ERK/P38 MAPK signaling pathways in HPDLCs

To investigate the mechanism involved in ferritin-induced cytokine release, Western blots were used. After HPDLCs were treated with different concentrations of apoferritin for 6 h, the phosphorylation of the ERK1/2, P38, and JNK MAPK pathways was assessed. The results showed that the ERK1/2 and P38 MAPK pathways were phosphorylated in this process, but apoferritin had no effect on the JNK/MAPK pathway (Figure 6). The maximum levels of pathway activation occurred at 1000 ng/ml (Figure 6A,B). Apoferritin induces the phosphorylation of MAP-kinase in a dose-dependent manner.

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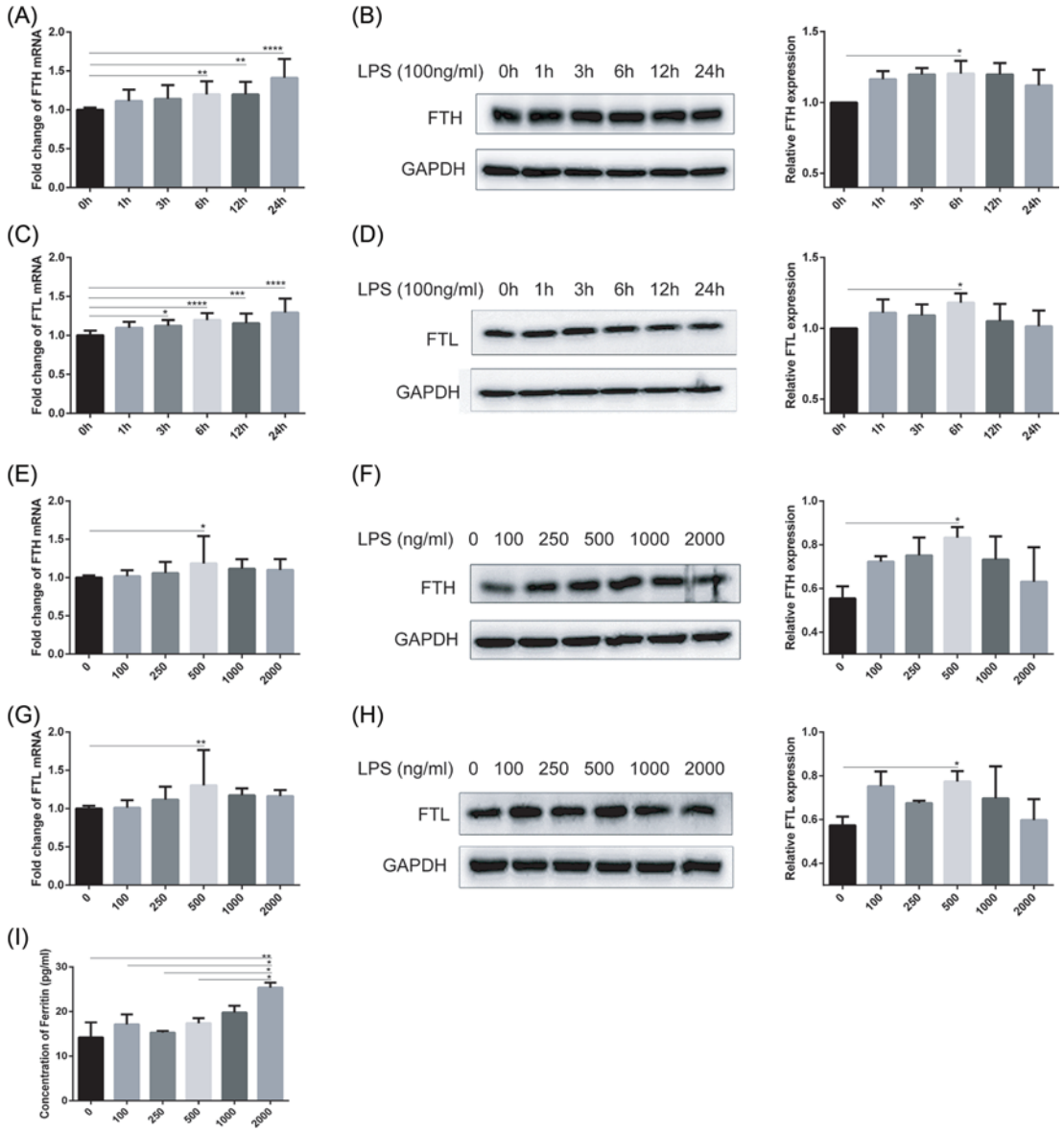

Figure 3. FTH and FTL are up-regulated dose- and time-dependently in HPDLCs after *P. gingivalis*-LPS stimulation

HPDLCs were treated with 100 ng/ml *P. gingivalis*-LPS for 0 to 24 h. (**A** and **C**) FTH and FTL mRNA rose to a peak at 24 h. (**B** and **D**) FTH and FTL protein levels peaked at 6 h. HPDLCs were treated with 0–2000 ng/ml *P. gingivalis*-LPS for 3 h. (**E** and **G**) FTH and FTL mRNA reached a peak at 500 ng/ml. (**F** and **H**) FTH and FTL protein levels also peaked at 500 ng/ml. HPDLCs were treated with 0–2000 ng/ml *P. gingivalis*-LPS for 6 h. (**I**) The secretion of ferritin was up-regulated after *P. gingivalis*-LPS stimulation, with one peak at 2000 ng/ml. Results are presented as the mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ compared with untreated cells.

To confirm the role of MAPKs in apoferritin-induced signaling in HPDLCs, we used the ERK1/2 inhibitor U0126 (10 μmol/l), the P38 inhibitor SB203580 (10 μmol/l), and the JNK inhibitor SP600125 (25 μmol/l). The increased expression of IL-6 and IL-8 induced by apoferritin was significantly reduced by the inhibitors of P38 and ERK at both the mRNA and protein levels, while the JNK inhibitor only suppressed their protein, but not their mRNA levels (Figure 7).

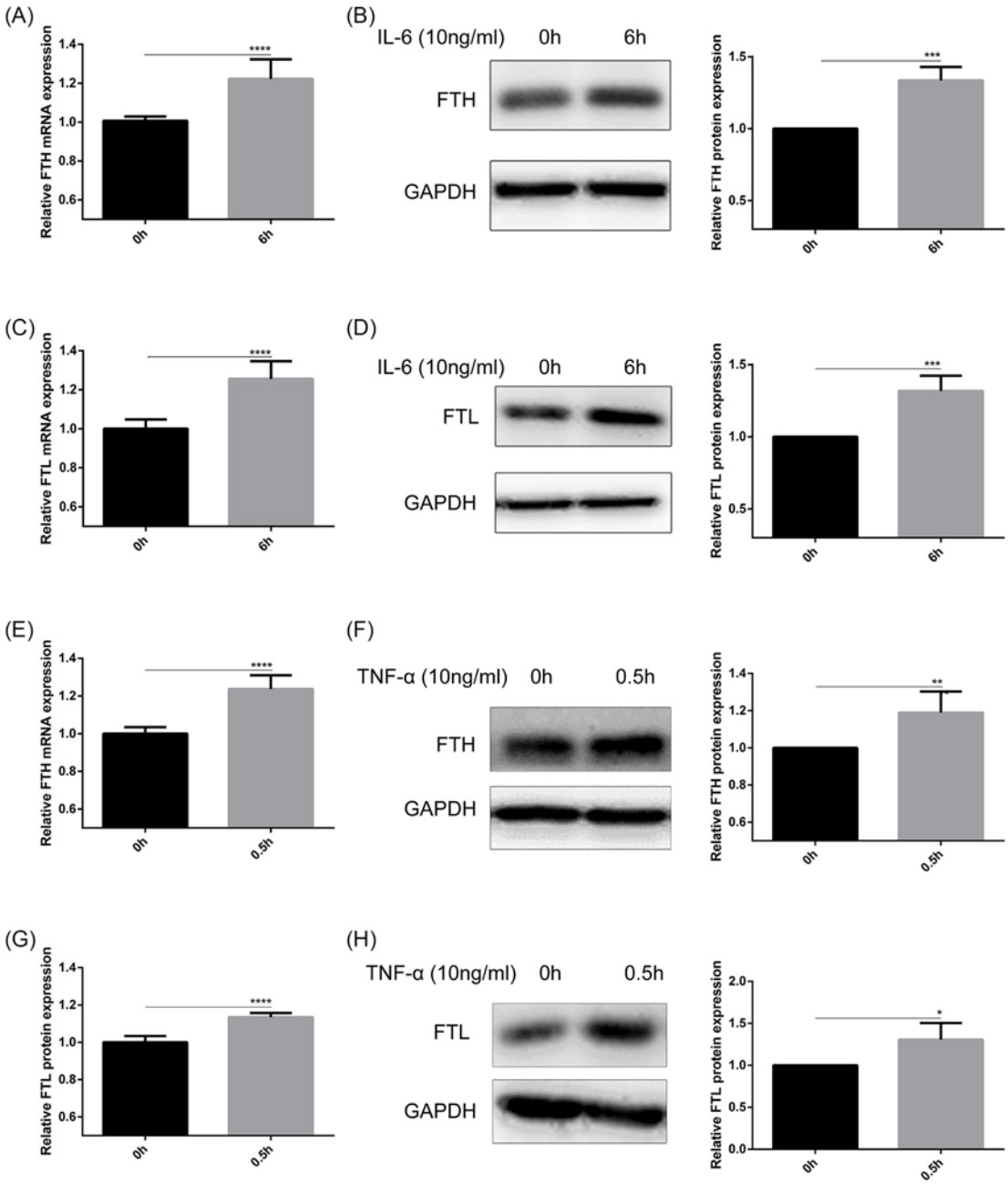

Figure 4. FTH and FTL are up-regulated in HPDLCs after IL-6 and TNF-α stimulation

HPDLCs were treated with 10 ng/ml IL-6 for 6 h (**A–D**) or 10 ng/ml TNF-α for 30 min (**E–H**). (A,C,E, and G) FTH and FTL mRNA expression were determined by real-time PCR. GAPDH was used as internal reference. (B,D,F, and H) Protein levels of FTH and FTL assessed by Western blot analysis using anti-FTH and anti-FTL antibodies. GAPDH was used to verify equal loading in each lane. Results are presented at the mean $\pm$ SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with untreated cells.

Ferritin promotes IL-6 and IL-8 production in HPDLCs partly via TfR-1

It has been reported that TfR-1 is a receptor for H-ferritin [31], although its role in initiating cell signaling is unknown. Here, knockdown of TfR-1 reduced the expression of IL-6 and IL-8 induced by 100 ng/ml apoferritin in HPDLCs (Figure 8), suggesting that apoferritin promotes inflammatory cytokine expression, at least in part, via TfR-1.

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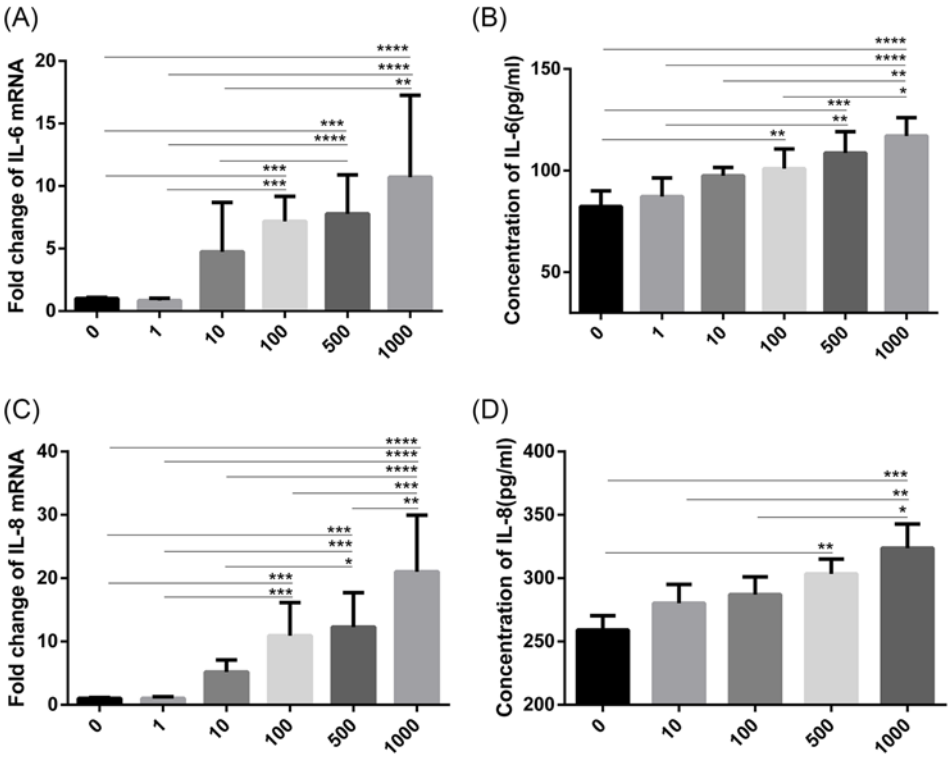

Figure 5. Ferritin induces IL-6 and IL-8 production in HPDLCs *in vitro*

HPDLCs were stimulated with different concentrations of apoferritin for 6 h. The mRNA levels of IL-6 (**A**) and IL-8 (**C**) were measured by qPCR, and the protein levels of IL-6 (**B**) and IL-8 (**D**) from cultured supernatants were assessed by ELISA. Results are presented at the mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 compared with the untreated cells.

Discussion

This is the first study to identify the specific correlation between ferritin and periodontitis. Our results demonstrated that: (1) ferritin is up-regulated by the inflammation in periodontitis and (2) exhibits cytokine-like activity to promote IL-6 and IL-8 responses in HPDLCs through TfR-1 via the ERK/P38 MAPK pathways, which may contribute to amplify the innate immune responses of periodontitis.

Periodontitis has become the leading cause of tooth loss in adults in the developing country. Unfortunately, we still don't know much about the mechanism of periodontitis. Pathogenic bacterium is the initiating factor of periodontitis, and the host's immunologic and inflammatory response to the bacteria can lead to periodontal destruction [5]. Therefore, research has been focused on host factors. According to analyses with a full-length cDNA library, ferritin is abundant in HPDLCs [25]. But its location and functions in periodontitis are unclear. In the present study, we investigated ferritin expression in periodontal tissues from humans and experimental mice. Interestingly, we found that ferritin was up-regulated in tissues affected by periodontitis, and the location of elevated expression correlated well with the extent of inflammatory infiltration (Figure 1). The expression of FTH and FTL were up-regulated along with aggravated inflammation (lymphocyte infiltration) that was confirmed using an animal model of experiment periodontitis (Figure 2). Ferritin, a ubiquitous iron-binding protein, sequesters iron in a non-toxic but bioavailable form and thus limits free radical generation triggered by inflammation [32–35]. Increased ferritin expression has been reported in atherosclerosis, cancer, and neurodegenerative diseases [15]. The increased ferritin may have cytoprotective function and anti-inflammatory effect in some cells [20,36]. However, further studies are needed in HPDLCs.

Ferritin regulation occurs at multiple levels [37,38]. Lipopolysaccharide and the pro-inflammatory cytokines TNFα, IL-1α, IL-1β, IL-6 modulate ferritin expression [39–43]. Previous studies show that the levels of *P. gingivalis*, IL-6, and TNF-α are higher in the pathogenic sites affected by periodontitis [44,45]. In the study, we found exogenous factors, such as *P. gingivalis*-LPS, are not the only factors of promoting ferritin expression (Figure 3), endogenous factors, such as IL-6 and TNF-α, also aggravate ferritin expression in HPDLCs (Figure 4). This is consistent with previous studies, which illustrate that several inflammatory cytokines could induce ferritin expression,

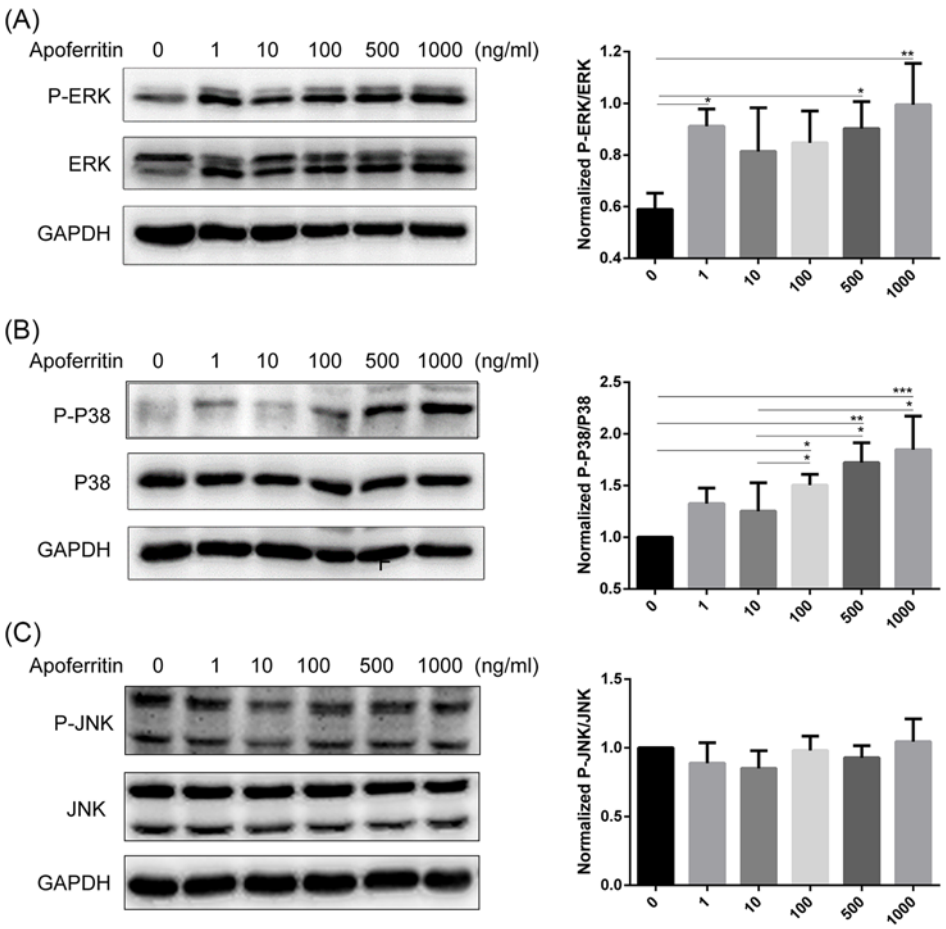

Figure 6. Apoferritin activated ERK/P38 MAPK signaling pathways in HPDLCs

After HPDLCs were treated with increasing concentrations of apoferritin for 6 h, cell lysates were collected. Phosphorylation of ERK1/2 (**A**), P38 (**B**) and JNK1/2 (**C**) MAPKs was analyzed by Western blot with specific antibodies as described in 'Materials and methods' section. The ratio of immunointensity between the phosphorylation of MAPKs (p-ERK1/2, p-P38, and p-JNK1/2) and total MAPKs (ERK1/2, P38, and JNK1/2) were calculated using ImageJ software. Both ERK (A) and P38 (B) were phosphorylated in a dose-dependent manner, but not JNK (C). Results are presented at the mean ± SEM. $^{*}P<0.05$, $^{**}P< 0.01$, $^{***}P<0.001$.

for example TNF-α, IL-1, and IL-6 [41,43,46]. These results suggested that the up-regulation of the ferritin gene and protein expression induced by pro-inflammatory cytokine in HPDLCs, which may be an early response of cells when they are stimulated by bacterium.

Surprisingly, although FTH expressed abundantly in the basal layer of healthy gingiva, it disappeared from the basal cell layer in inflammatory gingiva (Figure 1B,F). The reason for this phenomenon remains unclear. FTH and FTL are encoded by separate genes and have separable functions, and ferritin subunit composition can be readily modified in response to inflammatory and infectious conditions [15,47,48]. FTH possesses ferroxidase activity, converting Fe(II) to Fe(III) [15], is involved in iron-independent metabolisms, such as cell proliferation [15], angiogenesis, p53 regulation [49], chemokine transduction pathways [50], induction of epithelial to mesenchymal transition [51], and miRNAs expression [52], inhibiting hypoxia-inducible factor 1 [53]. The characteristic expression of FTH has little research at present. Further *in vitro* studies are needed to confirm the finding.

As reported, circulating ferritin level is elevated in systemic inflammation [23]. Furthermore, serum, salivary, and GCF ferritin are elevated in patients with chronic periodontitis [28,29,54]. However, the precise mechanisms of ferritin release and the functions of secreted ferritin in the serum and tissue are unclear [55]. T lymphocytes contribute to the synthesis and secretion of ferritin [56] and infiltrate in inflammatory periodontal tissues [57,58]. In the study, we found ferritin expression up-regulated in the area of T-lymphocytes infiltration. This may be a source of extracellular ferritin. Periodontal ligament cells, gingival fibroblasts, and gingival epithelial cells maintain a lot of ferritin. We

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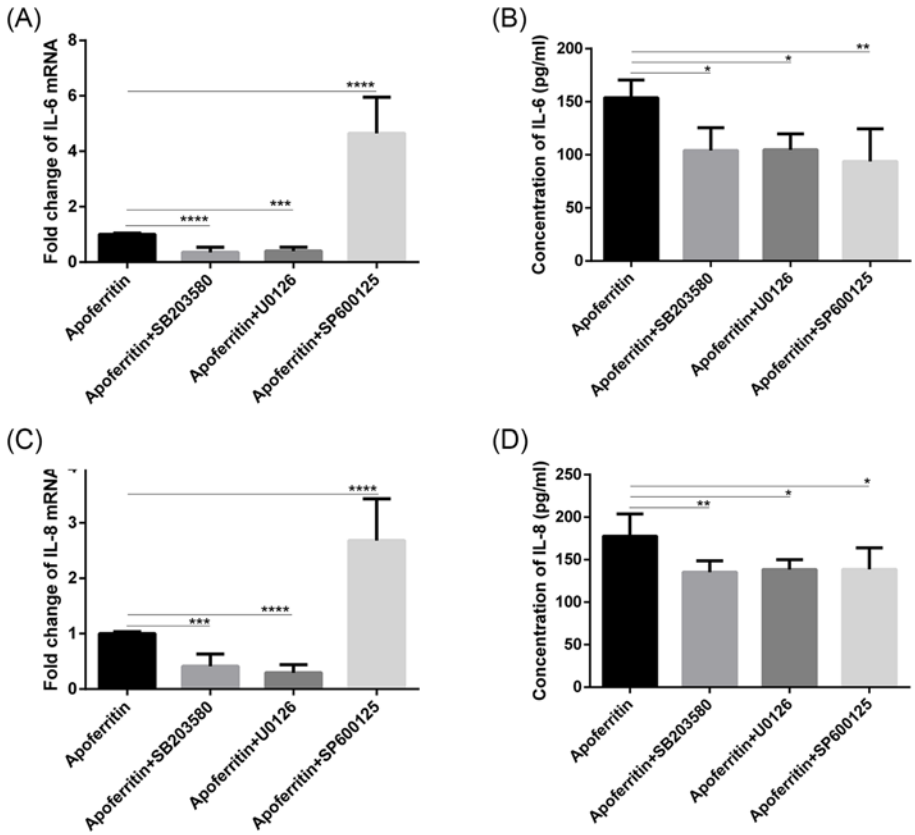

Figure 7. MAPK signaling pathways were involved in apoferritin-induced release of IL-6 and IL-8 from HPDLCs

HPDLCs were pre-incubated with the ERK1/2 inhibitor U0126 (10 μmol/l), the P38 inhibitor SB203580 (10 μmol/l), or the JNK inhibitor SP600125 (25 μmol/l) for 60 min before incubation with 100 ng/ml apoferritin in the continued presence of inhibitors. The increased expression of IL-6 and IL-8 induced by apoferritin were reduced by the inhibitors of P38 and ERK at both the mRNA (**A** and **C**) and protein (**B** and **D**) levels, while the inhibitor of JNK only suppressed the protein levels. Results are presented at the mean ± SEM. **P*<0.05, **P<0.01, ****P*<0.001, *****P*<0.0001 compared with the controls.

deduce that these cells may release ferritin when they are stimulated or damaged by bacteria. In the present study, we found *P. gingivalis*-LPS was a factor of promoting ferritin secretion (Figure 3). For the first time, our result demonstrated HPDLCs secreted ferritin.

Previously, studies report ferritin exist extracellularly, contain small amounts of iron [59] and act as a cytokine to regulate pro-inflammatory function in hepatic stellate cell via NF-κB-regulated signaling [55]. The results in the present study demonstrated that ferritin induces IL-6 and IL-8 production significantly in HPDLCs (Figure 5). IL-6 and IL-8 play important roles in the initiation and progression of periodontitis [7,20]. This suggest that ferritin may act as a second messenger system to pro-inflammation. That may amplify immuno-inflammatory responses in periodontal ligament of periodontitis.

The receptors and intracellular signaling pathway involved in ferritin-mediated IL-6 and IL-8 expression were further investigated. We found ferritin activated ERK and P38 MAPK pathways (Figure 6). The inhibitors of ERK and P38 also alleviated the pro-inflammatory effects of ferritin in the present study (Figure 7). Therefore, these results support the hypothesis that ferritin induced IL-6 and IL-8 production by mainly activating ERK and P38 MAPK pathways in HPDLCs.

Ferritin circulates and binds to a variety of cell types in a saturable manner. These cells include lymphocytes, brain oligodendrocytes, and erythroid precursors [60,61,62]. The identity of the ferritin receptor is unclear. It was reported that FTH interacts with cells through the TfR-1 and scavenger receptor class A member 5 [31,63,64]. Our results suggested that ferritin-induced release of IL-6 and IL8 from HPDLCs was partly dependent on TfR-1 (Figure 8). The role and the mechanism of ferritin as a modulator of periodontitis require further investigation.

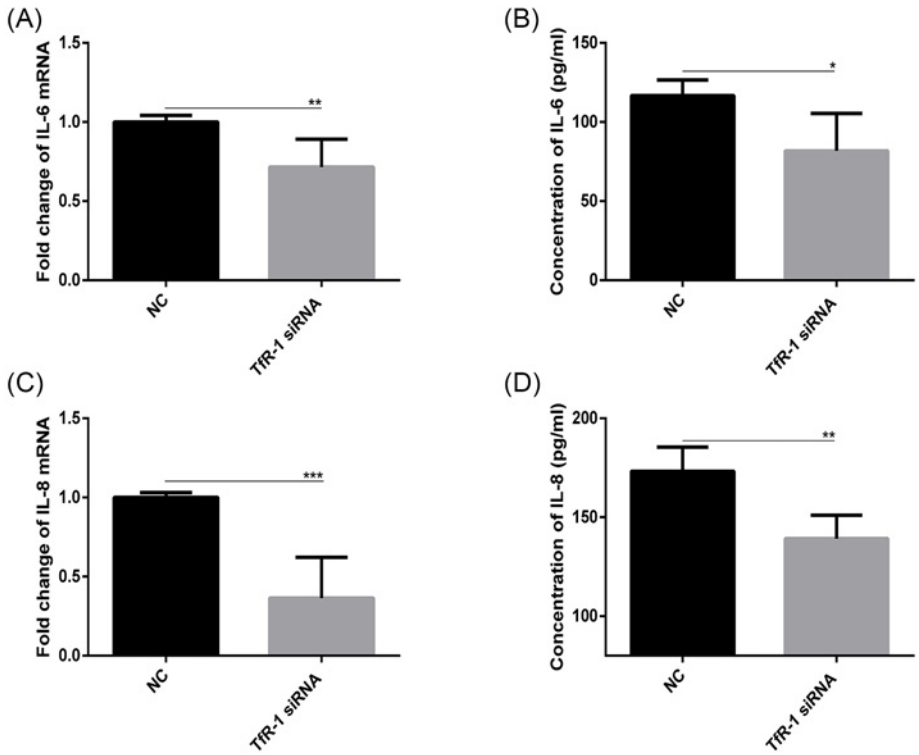

Figure 8. Effect of TfR-1 RNAi on IL-6 and IL-8 expression in HPDLCs after apoferritin simulation

After knockdown of TfR-1, the mRNA levels (**A** and **B**) and the protein levels (**C** and **D**) of IL-6 and IL-8 were reduced. Results are presented at the mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with controls.

Conclusion

The expression of ferritin is significantly positive in periodontal tissues of periodontitis. *P. gingivalis-LPS*, IL-6, and TNF-α, which are increased in patients of periodontitis, induce ferritin expression and secretion. Extracellular ferritin promotes pro-inflammatory cytokines production in HPDLCs, which may enhance the immune inflammatory responses in periodontitis. Taken together, ferritin may be involved in the development of periodontitis.

Clinical perspectives

- Periodontitis has become the leading cause of tooth loss in China. Unfortunately, we still don't know much about the mechanism of periodontitis. Ferritin is abundant in human PDL, and is closely related with inflammation.
- The expression of ferritin in periodontal tissues of periodontitis is elevated. Ferritin is up-regulated by *P. gingivalis*-LPS, IL-6, and TNF-α in HPDLCs. *P. gingivalis*-LPS also promotes the secretion of ferritin. And extracellular ferritin promotes pro-inflammatory cytokines production of HPDLCs. The transferrin receptor and ERK/P38 MAPK pathways involve in this process. Pro-inflammatory cytokines contribute to amplify the innate immune responses of periodontitis.
- Ferritin maybe a new biomarker and an attractive therapeutic target in patients of periodontitis.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

Wenxue Huang, contributed to design of the study, acquire the data, analysis the data, draft the manuscript; Yalin Zhan, Yunfei Zheng, Ye Han contributed to data acquisition and revise the article; Jianxia Hou, Wenjie Hu, contributed to the conception, design of the study, critically revise the manuscript. All authors have read and approved the final article.

Abbreviations

FTH, ferritin heavy polypeptide; FTL, ferritin light polypeptide; H&E, hematoxylin and eosin; HPDLC, human periodontal ligament cell; IL, interleukin; LPS, lipopolysaccharide; PDL, periodontal ligament; PDLC, periodontal ligament cell; TfR-1, transferrin receptor; TNF-α, tumor necrosis factor-α.

References

1 Reynolds, J.J. and Meikle, M.C. (1997) Mechanisms of connective tissue matrix destruction in periodontitis. *Periodontology* **14**, 144, https://doi.org/10.1111/j.1600-0757.1997.tb00195.x
2 Benedetto, A.D., Gigante, I., Colucci, S. and Grano, M. (2013) Periodontal disease: linking the primary inflammation to bone loss. *Clin. Dev. Immunol.* **2013**, 503754, https://doi.org/10.1155/2013/503754
3 Beertsen, W., Mcculloch, C.A. and Sodek, J. (1997) The periodontal ligament: a unique, multifunctional connective tissue. *Periodontology* **13**, 20–40, https://doi.org/10.1111/j.1600-0757.1997.tb00094.x
4 Jonsson, D., Nebel, D., Bratthall, G. and Nilsson, B.O. (2011) The human periodontal ligament cell: a fibroblast-like cell acting as an immune cell. *J. Periodontal. Res.* **46**, 153–157, https://doi.org/10.1111/j.1600-0765.2010.01331.x
5 Atsuo, A. (2010) Host–parasite interactions in periodontitis: microbial pathogenicity and innate immunity. *Periodontol 2000* **54**, 9–14, https://doi.org/10.1111/j.1600-0757.2010.00376.x
6 Hernández, I, M., Dutzan, N., García-Sesnich, J., Abusleme, L., Dezerega, A., Silva, N. et al. (2011) Host-pathogen interactions in progressive chronic periodontitis. *J. Dent. Res.* **90**, 1164, https://doi.org/10.1177/0022034511401405
7 El Awady, A.R., Messer RLGamal, A.Y., Sharawy, M.M., Wenger, K.H. and Lapp, C.A. (2010) Periodontal ligament fibroblasts sustain destructive immune modulators of chronic periodontitis. *J. Periodontol.* **81**, 1324, https://doi.org/10.1902/jop.2010.100054
8 Beertsen, W., McCulloch, C.A. and Sodek, J. (1997) The periodontal ligament: a unique, multifunctional connective tissue. *Periodontol 2000* **13**, 20–40, https://doi.org/10.1111/j.1600-0757.1997.tb00094.x
9 Ganga, G., Carla, M., Pearce, O.M.T., Reynolds, L.E., Kairbaan, H.D., Leinster, D.A. et al. (2015) Interleukin-6 stimulates defective angiogenesis. *Cancer Res.* **75**, 3098–3107, https://doi.org/10.1158/0008-5472.CAN-15-1227
10 Silva, T.A., Garlet, G.P., Fukada, S.Y., Silva, J.S. and Cunha, F.Q. (2007) Chemokines in oral inflammatory diseases: apical periodontitis and periodontal disease. *J. Dent. Res.* **86**, 306–319, https://doi.org/10.1177/154405910708600403
11 Zlotnik, A. and Yoshie, O. (2000) Chemokines: a new classification system and their role in immunity. *Immunity* **12**, 121–127, https://doi.org/10.1016/S1074-7613(00)80165-X
12 Su-Min, G., Ming, Z., Jian-Jun, H. and Jun-Zheng, W. (2009) Mitogen-activated protein kinases and phosphatidylinositol 3-kinase are involved in Prevotella intermedia-induced proinflammatory cytokines expression in human periodontal ligament cells. *Biochem. Biophys. Res. Commun.* **386**, 471–476, https://doi.org/10.1016/j.bbrc.2009.06.058
13 Lee, H.J., Cho, J.W., Kim, S.C., Kang, K.H., Lee, S.K., Pi, S.H. et al. (2006) Roles of p38 and ERK MAP kinases in IL-8 expression in TNF-α- and dexamethasone-stimulated human periodontal ligament cells. *Cytokine* **35**, 67–76, https://doi.org/10.1016/j.cyto.2006.07.009
14 Murayama, R., Kobayashi, M., Takeshita, A., Yasui, T. and Yamamoto, M. (2011) MAPKs, activator protein-1 and nuclear factor-kappaB mediate production of interleukin-1beta-stimulated cytokines, prostaglandin E(2) and MMP-1 in human periodontal ligament cells. *J. Periodontal. Res.* **46**, 568–575
15 Torti, F.M. and Torti, S.V. (2002) Regulation of ferritin genes and protein. *Blood* **99**, 3505–3516, https://doi.org/10.1182/blood.V99.10.3505
16 Theil, E.C. (1987) Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu. Rev. Biochem.* **56**, 289–315, https://doi.org/10.1146/annurev.bi.56.070187.001445
17 Arosio, P., Adelman, T.G. and Drysdale, J.W. (1978) On ferritin heterogeneity. Further evidence for heteropolymers. *J. Biol. Chem.* **253**, 4451–4458
18 Boyd, D., Vecoli, Charles, David, M and Belcher, S. K (1985) Structural and functional relationships of human ferritin H and L chains deduced from cDNA clones. *J. Biol. Chem.* **260**, 11755–11761
19 Caskey, J.H., Jones, C., Miller, Y.E. and Seligman, P.A. (1983) Human ferritin gene is assigned to chromosome 19. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 482–486, https://doi.org/10.1073/pnas.80.2.482
20 Fan, Y., Zhang, J., Cai, L., Wang, S., Liu, C., Zhang, Y. et al. (2014) The effect of anti-inflammatory properties of ferritin light chain on lipopolysaccharide-induced inflammatory response in murine macrophages. *Biochim. Biophys. Acta* **1843**, 2775–2783, https://doi.org/10.1016/j.bbamcr.2014.06.015
21 Zheng, L., Liu, Z., Wu, B., Dong, Y., Zhou, L., Tian, J. et al. (2016) Ferritin has an important immune function in the ark shell Scapharca broughtonii. *Dev. Comp. Immunol.* **59**, 15–24, https://doi.org/10.1016/j.dci.2015.12.010

22 Pham, C.G., Bubici, C., Zazzeroni, F., Papa, S., Jones, J., Alvarez, K. et al. (2004) Ferritin heavy chain upregulation by NF-kappaB inhibits TNFalpha-induced apoptosis by suppressing reactive oxygen species. *Cell* **119**, 529–542, https://doi.org/10.1016/j.cell.2004.10.017
23 Branco, R.G. and Garcia, P.C. (2017) Ferritin and C-reactive protein as markers of systemic inflammation in sepsis. *Pediatr. Crit. Care Med.* **18**, 194–196, https://doi.org/10.1097/PCC.0000000000001036
24 Khan, A., Khan, W.M., Ayub, M., Humayun, M. and Haroon, M. (2016) Ferritin is a marker of inflammation rather than iron deficiency in overweight and obese people. *J. Obes.* **2016**, 1937320, https://doi.org/10.1155/2016/1937320
25 Hou, J., Yamada, S., Kajikawa, T., Ozaki, N., Awata, T., Yamaba, S. et al. (2012) Role of ferritin in the cytodifferentiation of periodontal ligament cells. *Biochem. Biophys. Res. Commun.* **426**, 643–648, https://doi.org/10.1016/j.bbrc.2012.09.008
26 Hou, J., Yamada, S., Kajikawa, T., Ozaki, N., Awata, T., Yamaba, S. et al. (2014) Iron plays a key role in the cytodifferentiation of human periodontal ligament cells. *J. Periodontal. Res.* **49**, 260–267, https://doi.org/10.1111/jre.12103
27 Souvik, C., Shikha, T., Kumar, S.R. and Chander, N.S. (2014) Effect of non-surgical periodontal therapy on serum ferritin levels: an interventional study. *J. Periodontol.* **85**, 688–696, https://doi.org/10.1902/jop.2013.130107
28 Enhos, S., Duran, I., Erdem, S. and Buyukbas, S. (2009) Relationship between iron-deficiency anemia and periodontal status in female patients. *J. Periodontol.* **80**, 1750–1755, https://doi.org/10.1902/jop.2009.090209
29 Guo, L.N., Yang, Y.Z. and Feng, Y.Z. (2018) Serum and salivary ferritin and Hepcidin levels in patients with chronic periodontitis and type 2 diabetes mellitus. *BMC Oral Health* **18**, 63, https://doi.org/10.1186/s12903-018-0524-4
30 Yamada, S., Tomoeda, M., Ozawa, Y., Yoneda, S., Terashima, Y., Ikezawa, K. et al. (2007) PLAP-1/asporin, a novel negative regulator of periodontal ligament mineralization. *J. Biol. Chem.* **282**, 23070, https://doi.org/10.1074/jbc.M611181200
31 Li, L., Fang, C.J., Ryan, J.C., Niemi, E.C., Lebron, J.A., Bjorkman, P.J. et al. (2010) Binding and uptake of H-ferritin are mediated by human transferrin receptor-1. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 3505–3510, https://doi.org/10.1073/pnas.0913192107
32 Lawson, D.M., Treffry, A., Artymiuk, P.J., Harrison, P.M., Yewdall, S.J., Luzzago, A. et al. (1989) Identification of the ferroxidase centre in ferritin. *FEBS Lett.* **254**, 207–210, https://doi.org/10.1016/0014-5793(89)81040-3
33 Levi, S., Santambrogio, P., Cozzi, A., Rovida, E., Corsi, B., Tamborini, E. et al. (1994) The role of the L-chain in ferritin iron incorporation. Studies of homo and heteropolymers. *J. Mol. Biol.* **238**, 649–654, https://doi.org/10.1006/jmbi.1994.1325
34 Muhoberac, B.B., Baraibar, M.A. and Vidal, R. (2011) Iron loading-induced aggregation and reduction of iron incorporation in heteropolymeric ferritin containing a mutant light chain that causes neurodegeneration. *Biochim. Biophys. Acta* **1812**, 544–548, https://doi.org/10.1016/j.bbadis.2010.10.010
35 Santambrogio, P., Levi, S., Cozzi, A., Corsi, B. and Arosio, P. (1996) Evidence that the specificity of iron incorporation into homopolymers of human ferritin L- and H-chains is conferred by the nucleation and ferroxidase centres. *Biochem. J.* **314**, 139–144, https://doi.org/10.1042/bj3140139
36 Hatcher, H.C., Tesfay, L., Torti, S.V. and Torti, F.M. (2015) Cytoprotective effect of ferritin H in renal ischemia reperfusion injury. *PLoS One* **10**, e0138505 , https://doi.org/10.1371/journal.pone.0138505
37 Tsuji, Y., Ayaki, H., Whitman, S.P., Morrow, C.S., Torti, S.V. and Torti, F.M. (2000) Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. *Mol. Cell. Biol.* **20**, 5818, https://doi.org/10.1128/MCB.20.16.5818-5827.2000
38 Wasserman, W.W. and Fahl, W.E. (1997) Functional antioxidant responsive elements. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5361–5366, https://doi.org/10.1073/pnas.94.10.5361
39 Wang, L. (2012) Isolation and gene expression of yellow grouper ferritin heavy chain subunit after lipopolysaccharide treatment. *Biochem. Genet.* **50**, 467–475, https://doi.org/10.1007/s10528-011-9491-z
40 Torti, S.V., Kwak, E.L., Miller, S.C., Miller, L.L., Ringold, G.M., Myambo, K.B. et al. (1988) The molecular cloning and characterization of murine ferritin heavy chain, a tumor necrosis factor-inducible gene. *J. Biol. Chem.* **263**, 12638
41 Wei, Y., Miller, S.C., Tsuji, Y., Torti, S.V. and Torti, F.M. (1990) Interleukin 1 induces ferritin heavy chain in human muscle cells. *Biochem. Biophys. Res. Commun.* **169**, 289–296, https://doi.org/10.1016/0006-291X(90)91466-6
42 Miller, L.L., Miller, S.C., Torti, S.V., Tsuji, Y. and Torti, F.M. (1991) Iron-independent induction of ferritin H chain by tumor necrosis factor. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4946–4950, https://doi.org/10.1073/pnas.88.11.4946
43 Huang, S.N., Ruan, H.Z., Chen, M.Y., Zhou, G. and Qian, Z.M. (2018) Aspirin increases ferroportin 1 expression by inhibiting hepcidin via the JAK/STAT3 pathway in interleukin 6-treated PC-12 cells. *Neurosci. Lett.* **662**, 1–5, https://doi.org/10.1016/j.neulet.2017.10.001
44 Li, Y., Feng, X., Xu, L., Zhang, L., Lu, R., Shi, D. et al. (2015) Oral microbiome in chinese patients with aggressive periodontitis and their family members. *J. Clin. Periodontol.* **42**, 1015–1023, https://doi.org/10.1111/jcpe.12463
45 Zhan, Y., Lu, R., Meng, H., Wang, X., Sun, X. and Hou, J. (2017) The role of platelets in inflammatory immune responses in generalized aggressive periodontitis. *J. Clin. Periodontol.* **44**, 150–157, https://doi.org/10.1111/jcpe.12657
46 Kwak, E.L., Larochelle, D.A., Beaumont, C., Torti, S.V. and Torti, F.M. (1995) Role for NF-kappa B in the regulation of ferritin H by tumor necrosis factor-alpha. *J. Biol. Chem.* **270**, 15285–15293, https://doi.org/10.1074/jbc.270.25.15285
47 Harrison, P.M. and Arosio, P. (1996) The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta* **1275**, 161–203, https://doi.org/10.1016/0005-2728(96)00022-9
48 Hintze, K.J. and Theil, E.C. (2006) Cellular regulation and molecular interactions of the ferritins. *Cell. Mol. Life Sci.* **63**, 591–600, https://doi.org/10.1007/s00018-005-5285-y
49 Lee, J.H., Jang, H., Cho, E.J. and Youn, H.D. (2009) Ferritin binds and activates p53 under oxidative stress. *Biochem. Biophys. Res. Commun.* **389**, 399–404, https://doi.org/10.1016/j.bbrc.2009.08.125
50 Li, R., Luo, C., Mines, M., Zhang, J. and Fan, G.H. (2006) Chemokine CXCL12 induces binding of ferritin heavy chain to the chemokine receptor CXCR4, alters CXCR4 signaling, and induces phosphorylation and nuclear translocation of ferritin heavy chain. *J. Biol. Chem.* **281**, 37616–37627, https://doi.org/10.1074/jbc.M607266200

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https://doi.org/10.1042/CS20180679

51 Aversa, I., Zolea, F., Ieranò, C., Bulotta, S., Trotta, A.M., Faniello, M.C. et al. (2017) Epithelial-to-mesenchymal transition in FHC-silenced cells: the role of CXCR4/CXCL12 axis. *J. Exp. Clin. Cancer Res.* **36**, 104, https://doi.org/10.1186/s13046-017-0571-8
52 Di, M.S., Chirillo, R., Aversa, I., Biamonte, F., Santamaria, G., Giovannone, E.D. et al. (2018) shRNA targeting of ferritin heavy chain activates H19/miR-675 axis in K562 cells. *Gene* 657
53 Jin, P., Kang, J., Lee, M.K. and Park, J.W. (2018) Ferritin heavy chain controls the HIF-driven hypoxic response by activating the asparaginyl hydroxylase FIH. *Biochem. Biophys. Res. Commun.* **499**, 475–481, https://doi.org/10.1016/j.bbrc.2018.03.173
54 Naghsh, N., Sabet, N.K., Vahidi, F., Mogharehabed, A. and Yaghini, J. (2017) Relationship between periodontal disease and serum factors in patients undergoing hemodialysis. *Open Dent. J.* **11**, 701–709, https://doi.org/10.2174/1874210601711010701
55 Ruddell, R.G., Hoang-Le, D., Barwood, J.M., Rutherford, P.S., Piva, T.J., Watters, D.J. et al. (2009) Ferritin functions as a proinflammatory cytokine via iron-independent protein kinase C zeta/nuclear factor kappaB-regulated signaling in rat hepatic stellate cells. *Hepatology* **49**, 887–900, https://doi.org/10.1002/hep.22716
56 Dörner, M.H., Silverstone, A., Nishiya, K., De, S.A., Munn, G. and De, S.M. (1980) Ferritin synthesis by human T lymphocytes. *Science* **209**, 1019–1021, https://doi.org/10.1126/science.6967622
57 Cifcibasi, E., Ciblak, M., Kiran, B., Badur, S., Firatli, E., Issever, H. et al. (2015) The role of activated cytotoxic T cells in etiopathogenesis of periodontal disease: does it harm or does it heal? *Sci. Rep.* **5**, 9262, https://doi.org/10.1038/srep09262
58 Seymour, G.J. (1991) Importance of the host response in the periodontium. *J. Clin. Periodontol.* **18**, 421–426, https://doi.org/10.1111/j.1600-051X.1991.tb02310.x
59 Arosio, P., Ponzone, A., Ferrero, R., Renoldi, I. and Levi, S. (1986) Characteristics of ferritins in human milk secretions: similarities to serum and tissue isoferritins. *Clin. Chim. Acta* **161**, 201–208, https://doi.org/10.1016/0009-8981(86)90213-5
60 Hule, S.W., Heyliger, S.O., Powers, S. and Connor, J.R. (2000) Oligodendrocyte progenitor cells internalize ferritin via clathrin-dependent receptor mediated endocytosis. *J Neurosci Res.* **61**, 52–60, https://doi.org/10.1002/1097-4547(20000701)61:1<52::AID-JNR6>3.0.CO;2-T
61 Fargion, S., Arosio, P., Fracanzani, A., Cislaghi, V., Levi, S., Cozzi, A. et al. (1988) Characteristics and expression of binding sites specific for ferritin H-chain on human cell lines. *Blood* **71**, 753–757
62 Meyron-Holtz, E.G., Fibach, E., Gelvan, D. and Konijn, A.M. (1994) Binding and uptake of exogenous isoferritins by cultured human erythroid precursor cells. *Br. J. Haematol.* **86**, 635–641, https://doi.org/10.1111/j.1365-2141.1994.tb04797.x
63 Fargion, S., Fracanzani, A., Brando, B., Arosio, P., Levi, S. and Fiorelli, G. (1991) Specific binding sites for H-ferritin on human lymphocytes: modulation during cellular proliferation and potential implication in cell growth control. *Blood* **78**, 1056–1061
64 Li, J.Y., Paragas, N., Ned, R.M., Qiu, A., Viltard, M., Leete, T. et al. (2009) Scara5 is a ferritin receptor mediating non-transferrin iron delivery. *Dev. Cell* **16**, 35–46, https://doi.org/10.1016/j.devcel.2008.12.002